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PRINCIPAL INVESTIGATOR: Caroline D. Scatena, Ph.D.

CONTRACTING ORGANIZATION: Washington University
St. Louis, Missouri 63110

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St. Louis, Missouri 63110

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13. ABSTRACT *(Maximum 200 words)*

The hypothalamic neuropeptide hormone, Corticotropin Releasing Hormone (CRH), is also expressed in the placentas of humans and higher primates and may play an important role in the regulation of labor. In choriocarcinoma cell lines, which are models for placental trophoblasts, activation of cAMP dependent pathways increases human (h)CRH reporter gene expression. In addition to the cAMP response element (CRE) located at -220 bp in the human CRH promoter, a cAMP responsive region has been identified between -200 and -99 bp and a candidate 58 kDa transcription factor was identified in nuclear extracts of human, but not rodent, choriocarcinoma cell lines.

This region, which does not contain a canonical CRE, transfers protein kinase A responsiveness to a heterologous promoter. Electromobility shift assays and methylation and uracil interference studies localized factor binding to a 20 base pair region from -128 to -109 bp of the hCRH promoter. Base contacts identified in interference studies were confirmed as critical for binding, as a mutation of these bases abolished factor binding. Furthermore, a CRH promoter containing this mutation exhibited a diminished response to 8-Br-cAMP. These data identify this 58 kDa protein as the human-specific CRH activator previously identified as a candidate factor contributing to the species-specific expression of CRH in human placenta.

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Caroline P. Cotton 9/1/97

PI - Signature Date

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Introduction

Corticotropin releasing Hormone (CRH) is a 41 amino acid neuropeptide hormone which regulates the hypothalamic-pituitary-adrenal axis. It is highly conserved, and is primarily expressed in the parvocellular neurons of the paraventricular nucleus of the hypothalamus. Secretion of CRH into the hypophyseal portal blood system, activates the synthesis and secretion of ACTH from the anterior pituitary corticotrophs. ACTH, in turn, activates the synthesis and secretion of cortisol from the adrenal glands. Cortisol modulates this pathway via an inhibitory feedback loop, affecting both CRH and ACTH. Normally, CRH is expressed in a circadian pattern, however, during periods of stress CRH continues to be expressed, in spite of the high levels of circulating glucocorticoids (1). The regulation of this HPA pathway is primarily transcriptional. Some neuronal transcription factors, including the CREB/ATF and POU transcription factor families, have been identified as participating in the regulation of this complex neuroendocrine system (2,3,4,5,6). Yet, we still lack a complete understanding of the molecular mechanisms responsible for circadian expression, the stress response, and glucocorticoid feedback.

CRH is expressed at other sites in the central nervous system and in peripheral organs. In primates, the placenta produces the highest concentration of CRH outside of the hypothalamus (1), while other animal species, including rats, mice and guinea pigs fail to express CRH in their placenta (7,8). Recent studies indicate that placental CRH may serve as a key component in timing the onset of human labor (9). Placental CRH is identical to the peptide synthesized and secreted in the nervous system, and CRH is a single copy gene (1). Thus, the expression of placental CRH only in humans and high primates, and not in other species, indicates that unique mechanisms, distinct from those controlling hypothalamic expression, must control expression in placenta.

My previous studies have investigated the molecular mechanisms controlling this

species-specific placental expression of CRH. By comparing the activity of mouse and human CRH promoters in human and rodent trophoblast cell lines, I established that cellular differences, rather than DNA sequence differences, play the dominant role in establishing the species-specific expression pattern (10). In addition, a transgene containing the human CRH promoter, which was appropriately targeted and regulated in hypothalamus, was not expressed in mouse placenta (11). This confirmed the importance of species-specific *trans*-acting factors, rather than DNA sequence differences, in dictating the placental expression of CRH. Using nuclear extracts from the rodent and human cell lines, I identified three species-specific candidate *trans*-acting factors (10).

In my earlier studies, the ability to express CRH with both tissue and species-specificity appeared to be linked to cellular cAMP responsiveness. In addition to the highly conserved canonical CRE located at -220 bp (2,12), I identified an additional cAMP responsive region in the human CRH promoter from -200 to -99 bp, which does not contain a canonical cAMP regulatory site. One of my candidate *trans*-acting factors binds to this region and it is present only in nuclear extracts from the human choriocarcinoma cell lines (10). UV crosslinking identified a 58 kDa protein, present in human and not rodent trophoblasts, which represents the DNA binding component of the human-specific nuclear factor.

My current efforts have been to further characterize this protein factor and its DNA binding site, and to confirm its participation in placental expression of CRH. In this work, I present fine-mapping studies which identify a 20 base pair DNA binding site for the factor. I have confirmed the transcriptional activity of this factor on the CRH promoter, and its linkage to the cAMP responsiveness of the -200 to -99 bp region within the human CRH promoter.

Results

In Vitro DNA Binding Assays.

My previous studies in cell culture systems and in transgenic mice indicated that the expression of CRH in placenta is controlled by species-specific *trans*-acting factors (10,11). Transient transfections using deletions of the hCRH promoter identified a cAMP responsive regulatory region between - 200 and -99 bp, distinct from the canonical CRE at -220 bp. Examination of this region revealed that it did not contain a classical CRE. My candidate human-specific factor was present in nuclear extracts from human choriocarcinoma cell lines and bound to this region (10).

To more precisely define the binding site for this human-specific factor, nuclear extracts from JEG-3 cells were used in a series of electromobility shift assays (EMSA). The DNA fragments used in these analyses included portions of the hCRH proximal promoter spanning the region from -196 to -73 bp (FIG 1). Initially, two labeled fragments, -196 to -136 bp and -146 to -73 bp, were used to divide this region approximately in half. The candidate nuclear factor bound only to the fragment from -146 to -73 bp (10). Next, a series of unlabeled oligonucleotide pairs were used as competitors in EMSA with the -146 to -73 labeled hCRH fragment, to further define the location of the binding site. An oligonucleotide duplex from -146 to -107 bp of the hCRH promoter specifically competed the shifted band created by the candidate nuclear factor (FIG 1 and FIG 2A). Two other oligonucleotides, -117 to -98 bp and -112 to -73 bp, did not compete with the probe for the binding of the candidate nuclear factor (FIG 1 and FIG 2A). These competition experiments narrowed the binding site of the candidate nuclear factor to a region of the hCRH proximal promoter from -146 to -112 bp.

The -146 to -73 bp fragment of the hCRH promoter contains DraII sites centered at -130 and -129 bp (FIG 1). Digestion of this region with DraII generated two fragments for use in EMSA, but only the proximal fragment from -128 to -73 bp was bound by nuclear extracts (data

not shown). All of these results combined, narrowed the nuclear factor binding site to a 17 bp region from -128 to -112 within the hCRH promoter. A 17 bp oligonucleotide duplex was created which corresponds to this potential site. It was used in EMSA as both a labeled probe and as an unlabeled cold competitor for the -146 to -73 bp labeled fragment (FIG 1 and data not shown). The candidate nuclear factor bound this fragment, but only weakly. Also, this sequence was a weak and inconsistent competitor for binding of the factor in the presence of the -146 to -73 bp fragment (data not shown).

Because some of the fragments used in these studies may have split the binding site, the precise boundaries of the binding site were not certain. To more clearly identify the residues involved in binding, interference assays were performed.

Methylation and Uracil Interference Analysis.

In order to confirm and refine the DNA binding site identified by EMSA, methylation interference and uracil interference assays were performed. These assays identify DNA bases that, when modified, interfere with the binding of the nuclear factor to the hCRH promoter fragment (13). The labeled fragment used in these assays was the hCRH proximal promoter fragment from -146 to -73 base pair (FIG 1).

For the uracil interference analysis, end-labeled hCRH fragments were generated to contain partial substitutions of deoxyuracil for thymine residues. Because crude nuclear preparations contained nuclease activity that degraded deoxyuracil substituted DNA (S. Adler, unpublished results), binding reactions were performed using partially purified JEG-3 nuclear extracts. The results from these assays revealed that replacing the thymine with deoxyuracil at positions -121, -118, -116 and -114 on the sense strand, and at -111 on the anti-sense strand, interfered with the ability of the candidate nuclear factor to bind to the fragment (FIG 3A).

For the methylation interference analysis, end-labeled hCRH fragments were

partially methylated using dimethyl sulfate. These experiments identified the guanines at positions -120 and -113 on the sense strand, and at -112 on the anti-sense strand as residues critical for binding (FIG 3B).

These results from the interference assays clarified my initial mapping by EMSA. Interference assays identified the dA/T base pair at -111 of the hCRH proximal promoter as being important for candidate nuclear factor binding (FIG 3A). Deletion analysis did not have the resolution to clearly identify this base pair, and it is not included in the weakly active 17-mer fragment, -128 to -112 bp. Therefore, a 20-mer from -128 to -109 bp of hCRH was synthesized. The candidate nuclear factor bound strongly to this oligonucleotide (FIG 1 and data not shown). The 20-mer also effectively competed the bound factor from the -146 to -73 labeled hCRH fragment (FIG 1 and FIG 2B). Therefore, the DNA binding site for the human-specific nuclear factor is from -128 to -109 bp within the hCRH proximal promoter.

Mutation of the DNA binding site.

My identification of a minimal binding site for the human nuclear factor relied on *in vitro* binding studies. Prior to more functional studies, I performed site-directed mutagenesis to alter specific critical residues in the binding site (14). Two regions identified by interference studies were selected. The dT at -121 and the dG at -120 on the sense strand of hCRH were changed to dC at -121 and to dT at -120. The new mutated promoter fragment was named hCRH-200M1. The dG at -113 and the dC at -112 on the sense strand of hCRH were both changed to dA within the -200 hCRH proximal promoter fragment. The new mutated promoter fragment was named hCRH-200M2. These mutant hCRH fragments were used to generate labeled DNA fragments from -146 to -73 of the hCRH proximal promoter. The mutant probes were compared to the wild-type -146 to -73 hCRH fragment in an electromobility shift assay (FIG 4). The human-specific nuclear factor bound to the wild-type fragment and to the fragment with the M1 mutation, although binding was weak when compared to the wild-type fragment (FIG 4,

compare lane 2 with lane 3). Introducing the M2 mutation into the labeled fragment completely eliminated the ability of the nuclear factor to bind to its site (FIG 4, lane 4). These results were confirmed by using an oligonucleotide duplex from -128 to -109 of the hCRH promoter that contains the above mutations in EMSA as unlabeled cold competitors (data not shown). These analyses confirmed that the M1 mutation was a weak competitor while the M2 mutation was unable to compete for binding of the nuclear factor. These mutations thus confirm the identification of the binding site and the DNA base contacts critical for nuclear factor binding.

Transfection Studies.

The human-specific placental nuclear factor was initially identified in transient transfections as a participant in PKA mediated induction of the CRH promoter. This response was not dependent on the CRE sequence at -220 bp. My mapping has confirmed that binding of the nuclear factor is not dependent on the CRE, and the DNA binding site I identified does not resemble classical CRE's.

One of the characteristics of an independent regulatory element is the ability to transfer the sequence to a heterologous promoter and still retain the ability to activate gene expression. To determine if the cAMP responsive site fit this criterion, the oligo duplex from -146 to -107 bp within the hCRH promoter was multimerized in three and six copies, and inserted in front of a minimal 36 base pair promoter, p36 (15). When these reporter genes were transfected into the JEG-3 cells with the PKA catalytic subunit β, there was an activation of luciferase reporter gene expression (FIG 5A). The changes in expression from these multimerized constructs varied in multiple experiments from two to seven fold (data not shown). In figure 5A, the increase in expression due to three copies of the oligo was two fold when comparing to the p36 promoter, and this is equivalent to the change in expression seen with the -200 bp hCRH promoter. The increase in expression due to six copies was 5 fold when comparing to the activity of only the p36 promoter. The results from these experiments indicate that the region from -146 to -107 bp of the

hCRH promoter retains cAMP responsiveness when placed in front of a heterologous promoter.

The mutated promoters provide a way to further characterize the role of the placental nuclear factor by analyzing the functional consequences of factor binding on the placental expression of CRH. In transfection analysis, the activity of the CRH-200M2 promoter was compared to the activity of the native CRH-200 and CRH-99 bp promoters (FIG 5B). The mutant promoter has an approximately 50% decrease in reporter gene expression in response to activation of PKA pathways when compared to the wild-type CRH-200 bp promoter. The activation of reporter gene expression from the -200 bp promoter is relatively weak, and is approximately two fold greater than the induction seen with the herpes thymidine kinase control promoter. The mutation of the human-specific factor binding site results in a promoter with a PKA response midpoint between the response seen with the -200 and -99 bp CRH promoters.

Future Studies.

An attempt was made to clone the human-specific factor by Southwestern library screening and it was unsuccessful. It became apparent that the factor must be cloned by more traditional biochemical methods, or by screening a yeast one hybrid library. To clone the factor by these methods was not feasible during the time constraints of this proposal but will be attempted in future studies.

Specific Aim 2.

At the outset of these studies a second specific aim was proposed to examine the glucocorticoid regulation of CRH in human placenta. Initial transfection studies failed to demonstrate regulation of CRH by glucocorticoids in the choriocarcinoma cell lines (data not shown). The analysis of the species-specific expression of placental CRH identified three candidate *trans*-acting factors. In order to fully characterize these factors, my efforts were focused on their analysis, and I decided to discontinue my studies on the glucocorticoid regulation of CRH in the placenta.

Conclusions.

CRH is a single copy gene which is highly conserved and which displays a unique pattern of expression. Its primary site of expression is the hypothalamus, where it displays a circadian expression pattern, but one that is modulated by stress and by feedback regulation. It is also expressed in several peripheral tissues including placenta (1). The expression in placenta is uniquely species-specific. Only humans and high primates express the gene in this organ (7). This placental expression may reflect the unique changes to fetal gestation and parturition that have occurred in human evolution. Furthermore, the mechanisms controlling the expression of CRH in the placenta must be distinct from those controlling the expression of CRH in the HPA axis, which are highly conserved across many species (1).

One other placental gene that has been extensively studied is the alpha subunit of the glycoprotein chorionic gonadotropin. It has a central neuroendocrine role as an anterior pituitary peptide. It also has a species-specific expression pattern in placenta, with expression in primates and horses. For α-CG, evolutionary changes in *cis*-acting sequences within the promoter of the gene dictate the species-specific expression in placenta. The presence of one or two copies of a CRE is essential for α-CG expression and minor changes in this *cis*-acting element results in the loss of expression in placenta (16,17,18). In addition, the *cis*-acting sequence, TSE (tissue specific element), contributes to the tissue-specific expression of α-CG (18,19,20). Experiments in transgenic mice have shown that the bovine α-CG promoter is expressed only in pituitary, while a transgene derived from the human promoter is expressed both in pituitary and placenta (17). It is therefore a combination and alteration of these *cis*-acting elements, and not differences in *trans*-acting factors, that play the dominant role in species-specific expression of α-CG in placenta.

My studies present a different paradigm to explain the species-specific expression

of CRH in placenta. Transfection studies identified three regions within the human CRH promoter that contribute to the expression pattern of CRH within the placental trophoblast. *In vitro* studies identified candidate nuclear factors binding to the regions targeted by my transfection analyses. Interestingly, these nuclear factors are species-specific; a candidate rodent repressor and activator were identified in rodent trophoblasts and a candidate human-specific factor was identified in human cells (10). The conclusion from these *in vitro* studies was confirmed *in vivo* using a transgenic mouse model. We detected regulated expression of a hCRH transgene in the hypothalamus, but I was unable to detect expression of the transgene in placenta (11). These *in vivo* results confirmed my *in vitro* findings and demonstrated differences in *trans*-acting factors, not *cis*-acting sequences, dominate in determining the expression of CRH in placental trophoblasts.

In human trophoblast cells, cAMP plays a critical role in both cell differentiation and gene expression (21,22). Many genes in these cells, including both α-CG and CRH, are regulated by cAMP (2,3,23,24). The results from my earlier studies implied that it was the ability of a cell to respond to cAMP which determines both the tissue and species-specific expression of CRH (10). CRH has within its proximal promoter a highly conserved classical CRE where members of the CREB/ATF family of transcription factors can bind and activate CRH gene expression (2,12,25). My studies indicated that after mutational inactivation of the canonical CRE, cAMP responsiveness was retained. Deletional mapping identified a proximal cAMP responsive region within the human CRH promoter from -200 to -99 bp, which does not contain any characterized classical cAMP response elements (10). The human-specific factor binds to hCRH within this region and fine mapping studies defined its binding site to be from -128 to -109 bp within the hCRH promoter. In my current studies, transfection experiments using human choriocarcinoma cell lines were performed to determine the role the factor plays in modulating the cAMP responsiveness of the -200 to -99 bp region. In these analyses, the region from -146 to -107 bp of hCRH was transferred to a heterologous promoter, and the DNA binding site for the

human-specific factor was mutated in the context of the hCRH promoter.

In the transfection studies that involved transfer of the region to a heterologous promoter, the site retained cAMP responsiveness. In these studies, the ability to activate reporter gene expression, when either three or six copies of the element were inserted in front of a minimal promoter, varied from two to seven fold above the activity seen with the p36 promoter alone. This variability can be attributed to a few possibilities. The 5' and 3' end of the -146 to -107 oligo duplex is fairly GC rich, and multimerization of these oligos may result in changes in DNA structure that are not compatible with efficient transfection analysis. There also exists the possibility that other factors may be involved in the cAMP responsiveness of the -200 to -99 bp region. The binding site for these factors may have been disrupted or even removed in these constructs. In addition, the growth conditions of the cells may affect the expression and activity of any additional factors involved in the cAMP regulation of this region. To more precisely define the role the human-specific factor plays in the modulation of the cAMP responsiveness of the -200 to -99 bp region, the DNA binding site for the factor was disrupted in the context of the -200 bp hCRH promoter. In transfection studies, the M2 mutation still retained the ability to activate reporter gene expression 50% in response to PKA pathways, even though the human-specific factor can not bind to this mutated promoter *in vitro*. Majzoub, et al. also have identified a PKA responsive site which they believe resides between -112 and -98 bp in the hCRH promoter (26). This site was identified by its similarity to a site found in the human enkephalin promoter (27).[1] The site is adjacent to the human-specific factor DNA binding site at -128 to -109 bp in the hCRH promoter, and the two sites overlap by four base pairs. Perhaps, it is the combination of both of these sites that contributes to the cAMP responsiveness of this region. In my footprinting analysis, we did not detect binding of a factor to the -117 to -103 bp region, but the extract used in this

[1] The sequence presented in the publication differs from the actual CRH sequence by a single nucleotide at -109. The change does not significantly alter the observed homology.

analysis was partially purified and selected for enrichment of the human-specific factor. It is possible that in crude nuclear extract a footprint might be detected in the -117 to -103 bp region. In addition, my earlier studies indicated the possibility that other distal PKA regulated sites exist within the 5 Kb human CRH promoter (10). The region from -200 to -99 bp contains the most proximal site we could detect by deletion analysis, and may not be the best binding site for the factors mediating the PKA responsiveness of the hCRH promoter. The combination of several cAMP regulated sites including the CRE, as well as the absence of transcriptional repressors, may ultimately contribute to the tissue and species-specific expression of CRH, as well as the regulated responses of CRH to environmental or developmental signals.

The unique placental expression of CRH in higher primates is consistent with a role for CRH in fetal gestation and labor, especially in humans (28). The expression of CRH in human placenta begins around the seventh week of gestation, and increases throughout pregnancy. During the last five weeks of gestation, there is a significant increase in CRH expression within the placenta (29). Studies have correlated placental expression of CRH with the length of gestation. Elevations of CRH occur earlier in pregnancies complicated by pre-term delivery, and the level of CRH is lower in pregnancies extending post-term (9). Placental CRH may also cross into the fetal circulation and stimulate the fetal HPA axis, resulting in the increase in cortisol seen within the fetus during the last 5 weeks of gestation. The cortisol surge is necessary for the maturation of fetal organs, and thus may contribute to the fetal signal for initiating parturition (30).

In my model cell culture system, the activity of the human-specific factor, in both specific DNA binding and mediation of a transcriptional response to cAMP, varies with growth conditions and cell density (M.A.M. and C.D.S. unpublished results). It is tempting to speculate that these changes in activity parallel the changes occurring in the trophoblast at term that result in the increased expression of CRH in human placenta. The role CRH plays in fetal gestation and parturition suggests a requirement for strict regulation of the CRH gene in placenta. Further

characterization of this protein, including cloning of its gene and determination of the role cAMP plays in its expression and activity, could clarify the mechanisms and role of CRH in the human placenta and in parturition.

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Appendix.

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All research summarized in this Annual Report has been submitted for publication in a peer-reviewed journal. In keeping with generally accepted principles and specific journal requirements, we have promised that as a pre-condition for publication, **no other publicity or distribution, including reports in the lay press, may precede the journal publication of our complete reports.** The data contained in this annual report may be distributed on a confidential basis to enable appropriate review and evaluation for continued funding. Any other distribution should be postponed until after journal publication.

Figure Legends

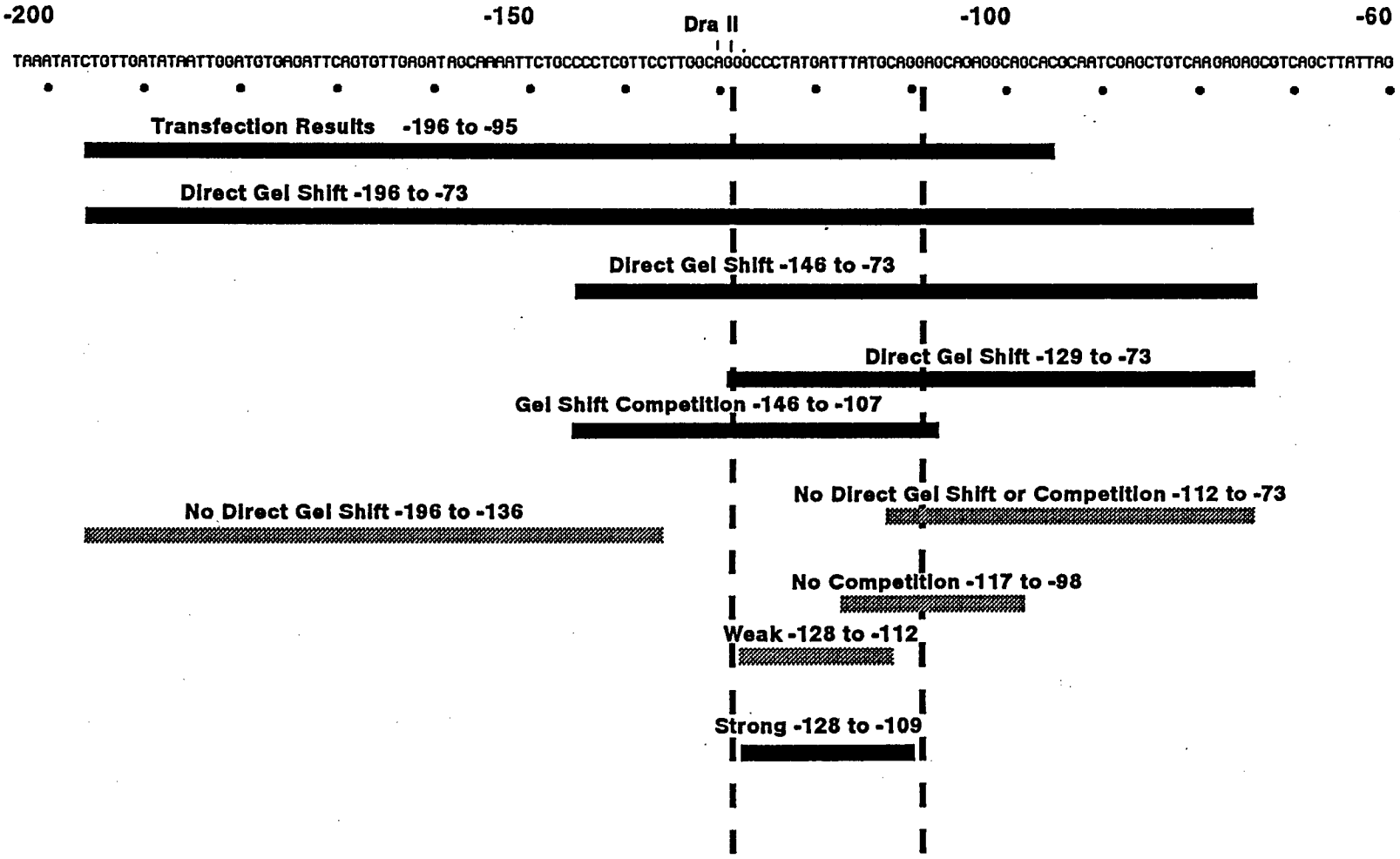
Figure 1. Location of the Human-Specific Nuclear Factor DNA Binding Site. The DNA sequence for the human CRH promoter from -200 to -60 bp is shown for reference. Results obtained from transfection and electromobility shift analyses are indicated for each fragment. The 20 bp binding site identified by these analyses is from -128 to -109 bp in the hCRH promoter, and is **dGGCCCTATGATTTATGCAGG**.

Figure 2. Competition Studies Map the Location of the Human-Specific Nuclear Factor DNA Binding Site. The labeled fragment used in these electromobility shift assays is from -146 to -73 bp in the hCRH promoter. The arrow indicates the location of the complex. **A.** Competition with hCRH promoter fragments. JEG-3 nuclear extract was incubated with the labeled probe in the absence or presence of excess (1 pmol) unlabeled fragments. P: probe only; Lanes 1-5: probe and nuclear extract with no competitor (lane 1); hCRH -146 to -73 as competitor (lane 2); hCRH -146 to -107 as competitor (lane 3); hCRH -117 to -98 as competitor (lane 4); hCRH -112 to -73 as competitor (lane 5). **B.** Competition with 20-mer binding site from -128 to -109 bp in the hCRH promoter. JEG-3 nuclear extract was incubated with the labeled probe in the absence or presence of excess unlabeled 20-mer fragment. P: probe only; N: no competitor; Crescendo: increasing quantities (1 pmol, 3 pmol, 6 pmol) of unlabeled 20-mer fragment (lane 3-5).

Figure 3. Uracil Interference and Methylation Interference. Partially purified JEG-3 nuclear extract was incubated with the hCRH fragment from -146 to -73 bp which had been end labeled on either the sense or anti-sense strand. **A.** dU Interference. The fragments were generated to contain partial substitution of deoxyuracil for thymidine residues. The DNA bases that when modified affected the binding of the protein are noted with arrows, and their location in the hCRH promoter is given. Free, probe not bound by extract; Bound, probe bound by extract in EMSA. The end-labeled strands are either sense or anti-sense, as indicated. **B.** Methylation interference. The fragments were partially methylated using dimethyl sulfate. The DNA bases that when modified affected the binding of the protein are noted with arrows, and their location in the hCRH promoter is given. Free, probe not bound by extract; Bound, probe bound by extract in EMSA. The end-labeled strands are either sense or anti-sense, as indicated.

Figure 4. Mutation of the Human-Specific Factor DNA binding site alters binding of the protein in electromobility shift assays. The labeled fragments used in these electromobility shift assays were -146 to -73 of the hCRH promoter (wild type, M1, or M2) and were incubated with JEG-3 nuclear extract, as indicated. The **M1** mutation contains a change in the hCRH nucleotides at positions -121 and -120 from dTG to **dCT**. The **M2** mutation contains a change in the hCRH nucleotides at positions -113 and -112 from dGC to **dAA**. The arrow indicates the location of the complex. P, wild type probe alone; WT, wild type probe with nuclear extract; M1, probe with M1 mutation with nuclear extract; M2, probe with M2 mutation with nuclear extract.

Figure 5. Analysis of the Human-Specific Factor and the cAMP response of the -200 to -99 bp region of hCRH in human choriocarcinoma cell lines. Human JEG-3 cells were transfected with the indicated reporters and harvested for luciferase assays. **A.** Transfer of cAMP responsiveness to a heterologous promoter. Cells were transfected with the indicated reporter and either RSV-Neo or RSV-PKA catalytic subunit ß . Fold expression is the relative increase in luciferase activity due to the expression and activity of the PKA catalytic subunit ß for each promoter construct, compared to the RSV-Neo control. MULTI 3 and MULTI 6 are p36 minimal promoters with three and six copies of the -146 to -107 hCRH oligonucleotide, respectively. Results shown are from one experiment, and the experiment was repeated three times with similar results. **B.** Mutation of the human-specific factor DNA binding site in the context of the -200 bp hCRH proximal promoter. Cells were treated with 8-Br-cAMP for 24 hrs prior to harvesting for luciferase assays. Fold expression is the relative increase in luciferase activity with hormone treatment for each promoter construct. Results displayed are the means $\pm$ SEM (n=4) except for the TK (n=3).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-150
Dra II
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-60
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Transfection Results -196 to -95
Direct Gel Shift -196 to -73
Direct Gel Shift -146 to -73
Direct Gel Shift -129 to -73
Gel Shift Competition -146 to -107
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No Direct Gel Shift -196 to -136
No Competition -117 to -98
Weak -128 to -112
Strong -128 to -109

Figure 1

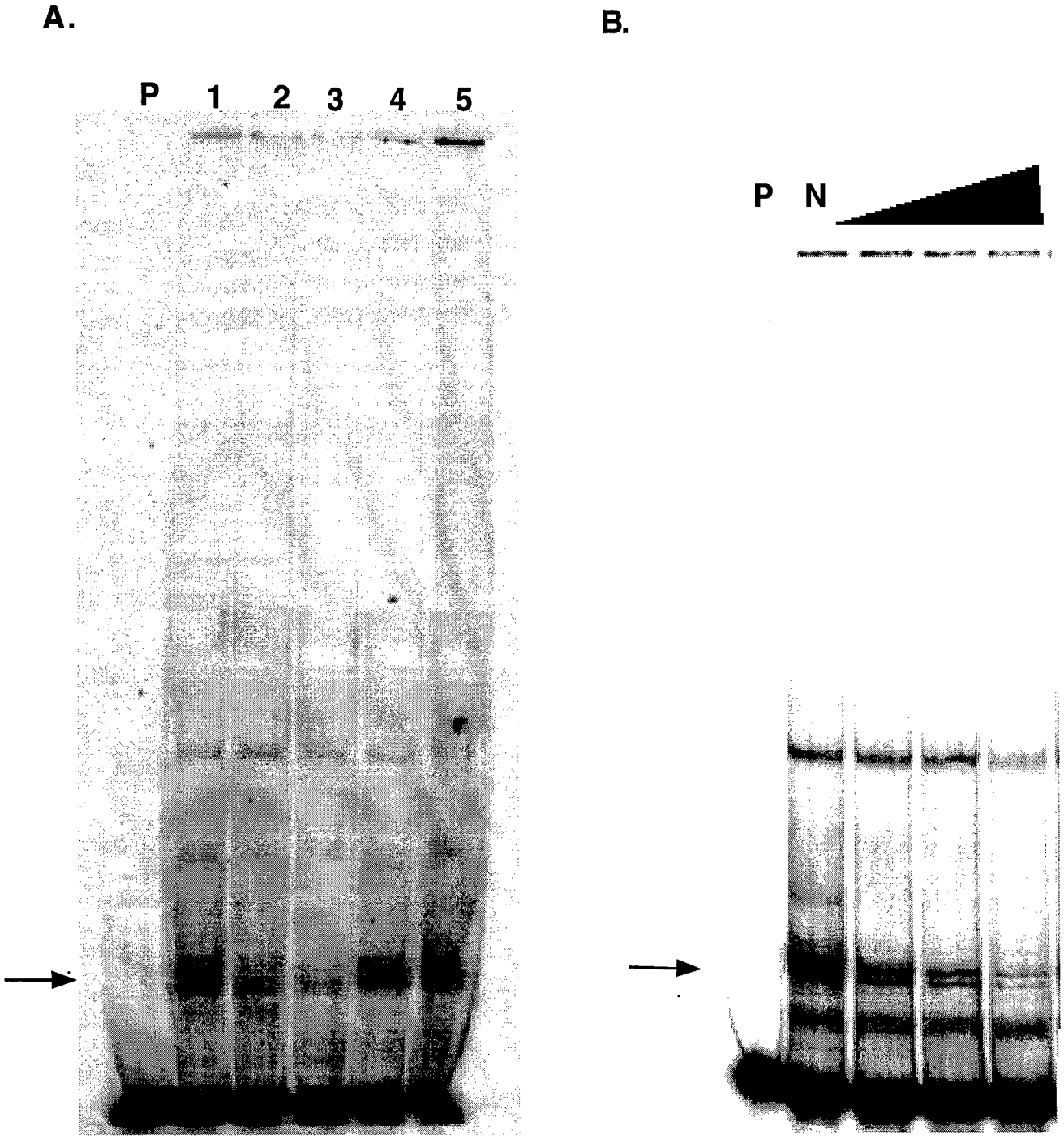


Figure 2

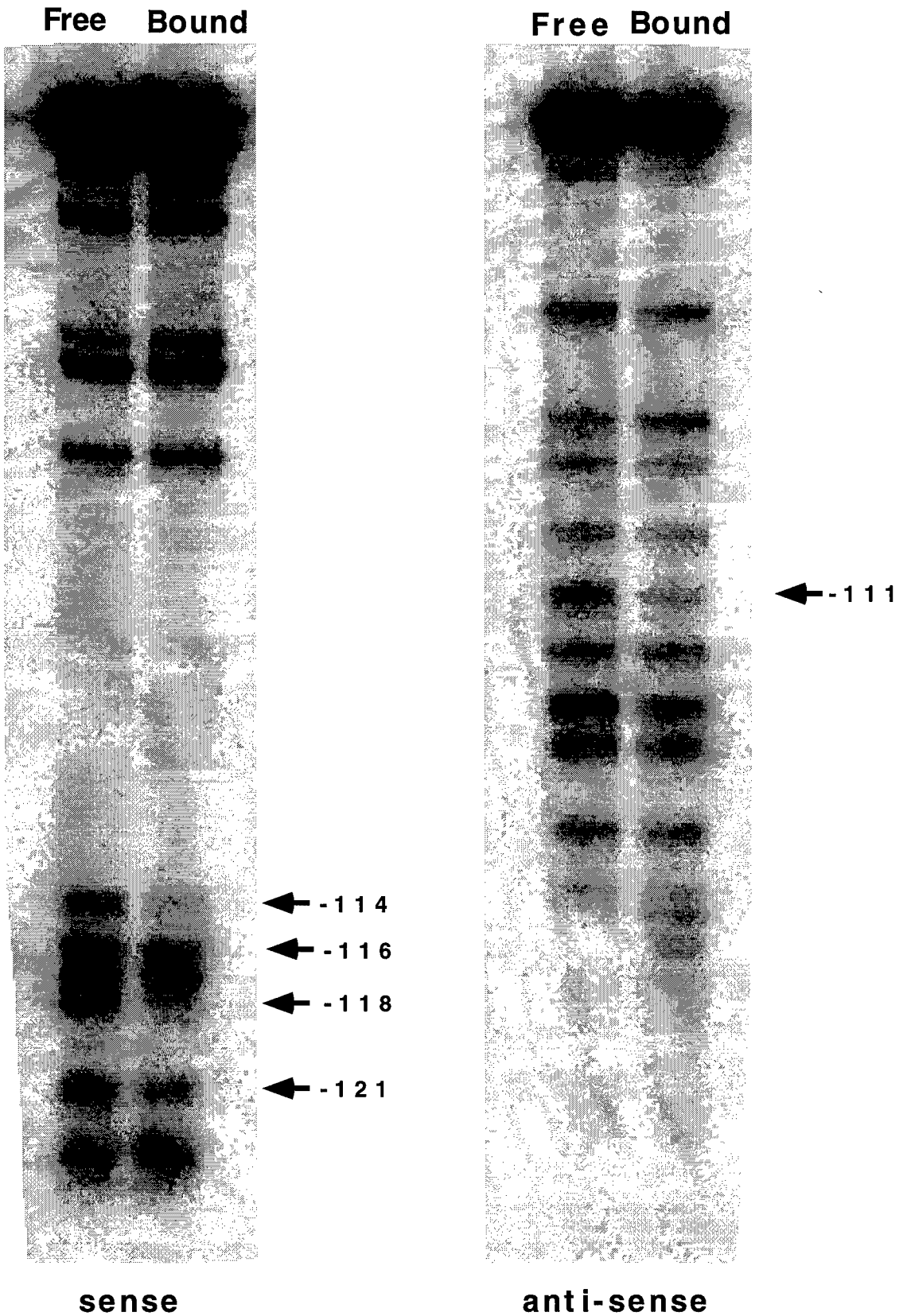

dU Interference
Free Bound
-114
-116
-118
-121
sense
Free Bound
-111
anti-sense

Figure 3A

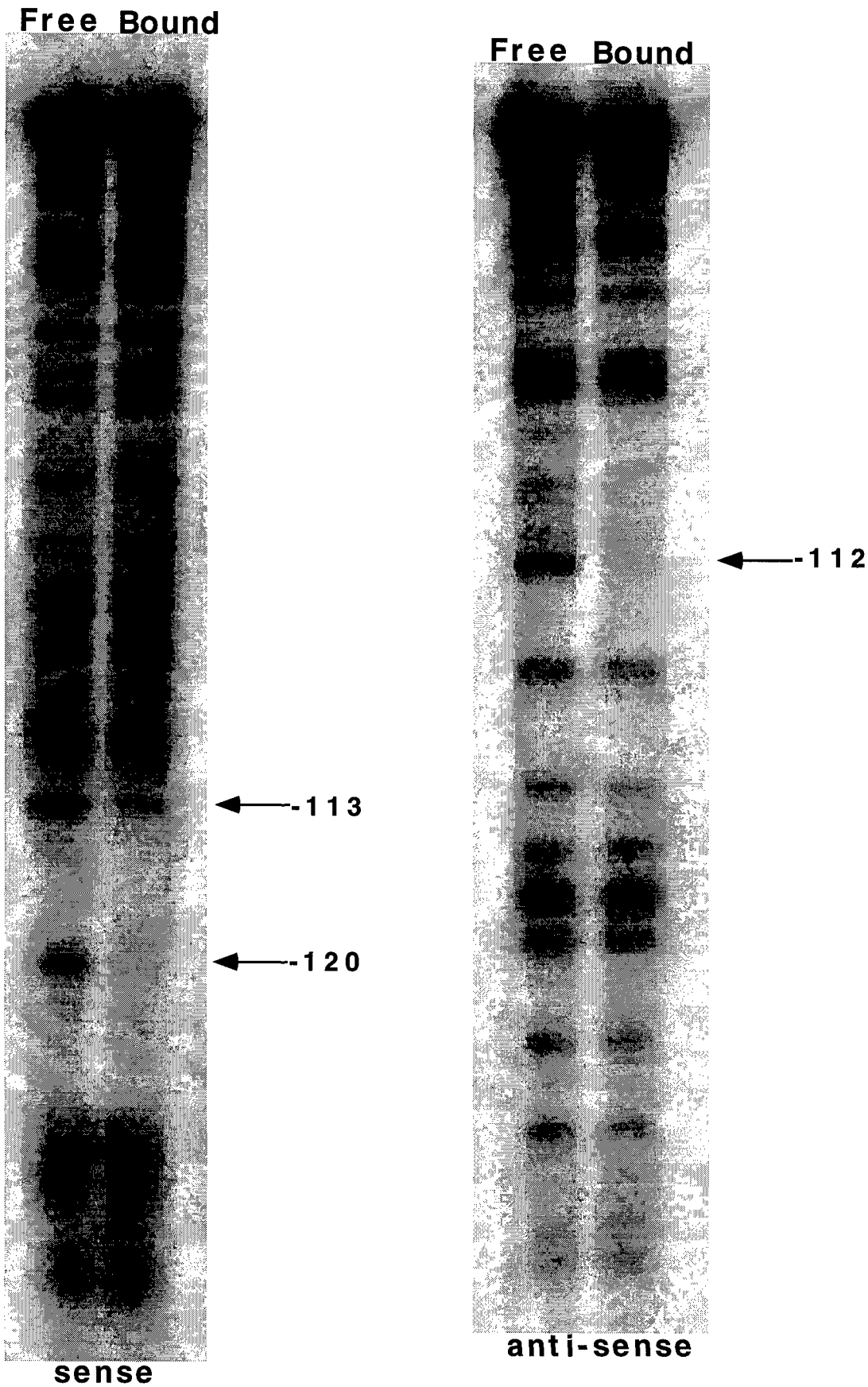

Methylation Interference
Free Bound
-113
-120
sense
Free Bound
-112
anti-sense

Figure 3B

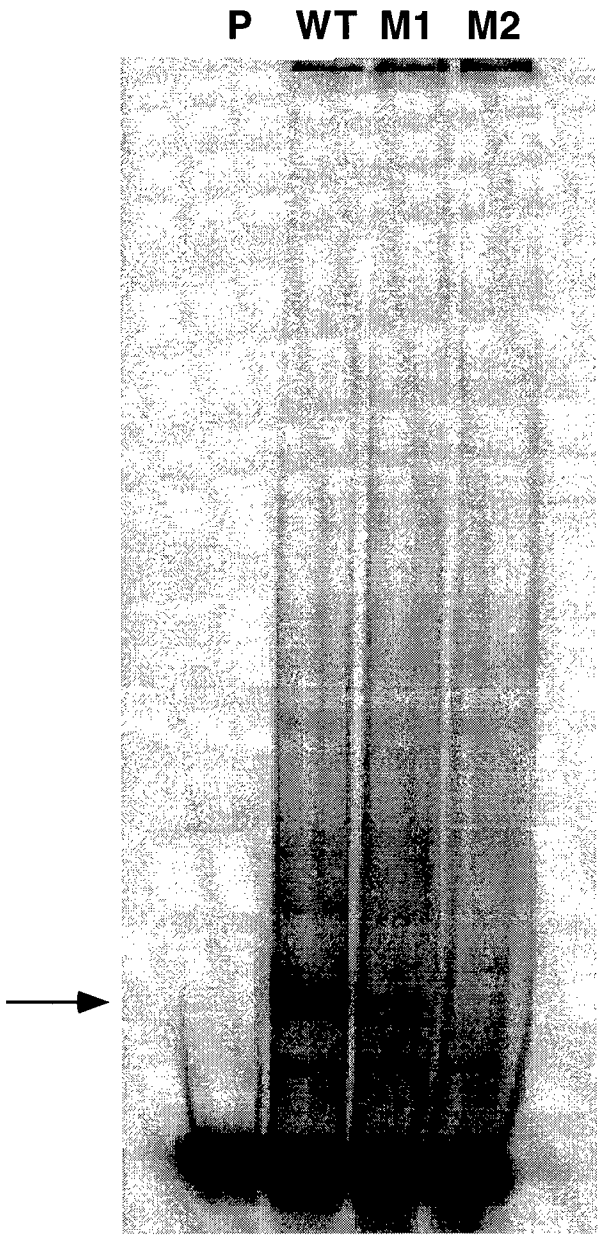
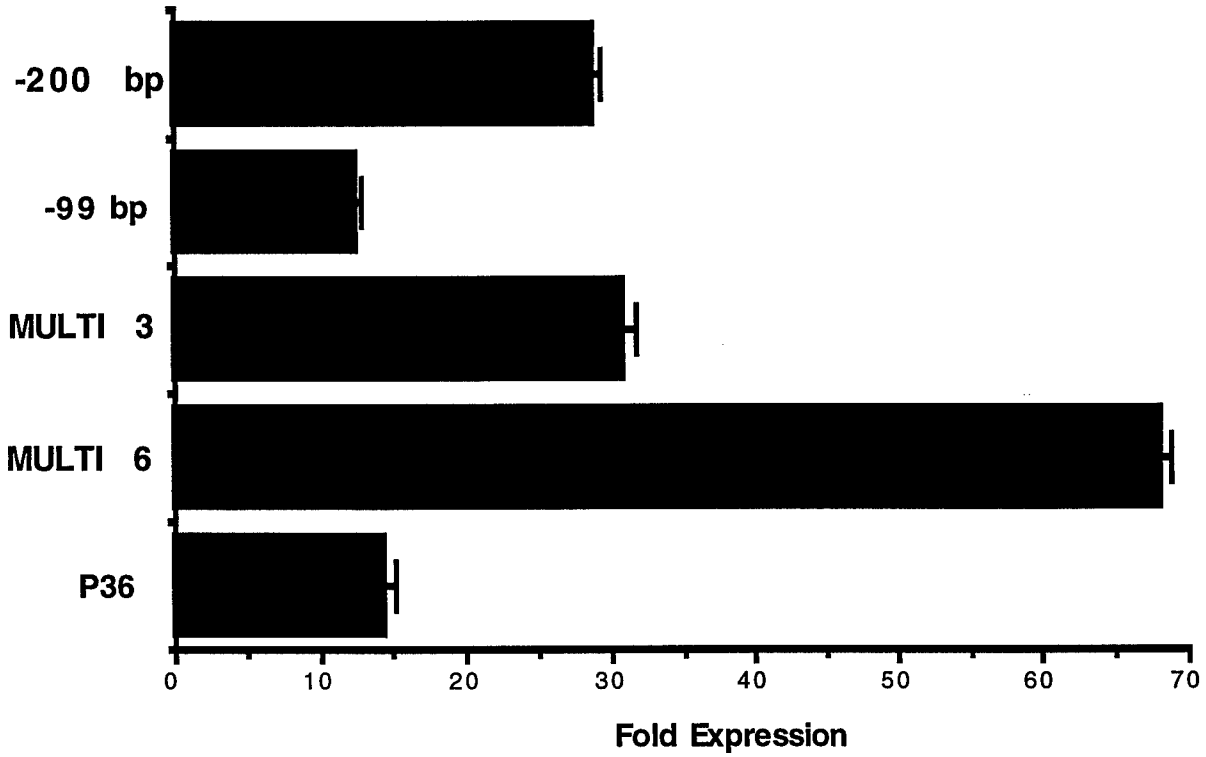


Figure 4

A.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-99 bp
MULTI 3
MULTI 6
P36
0
10
20
30
40
50
60
70
Fold Expression

B.

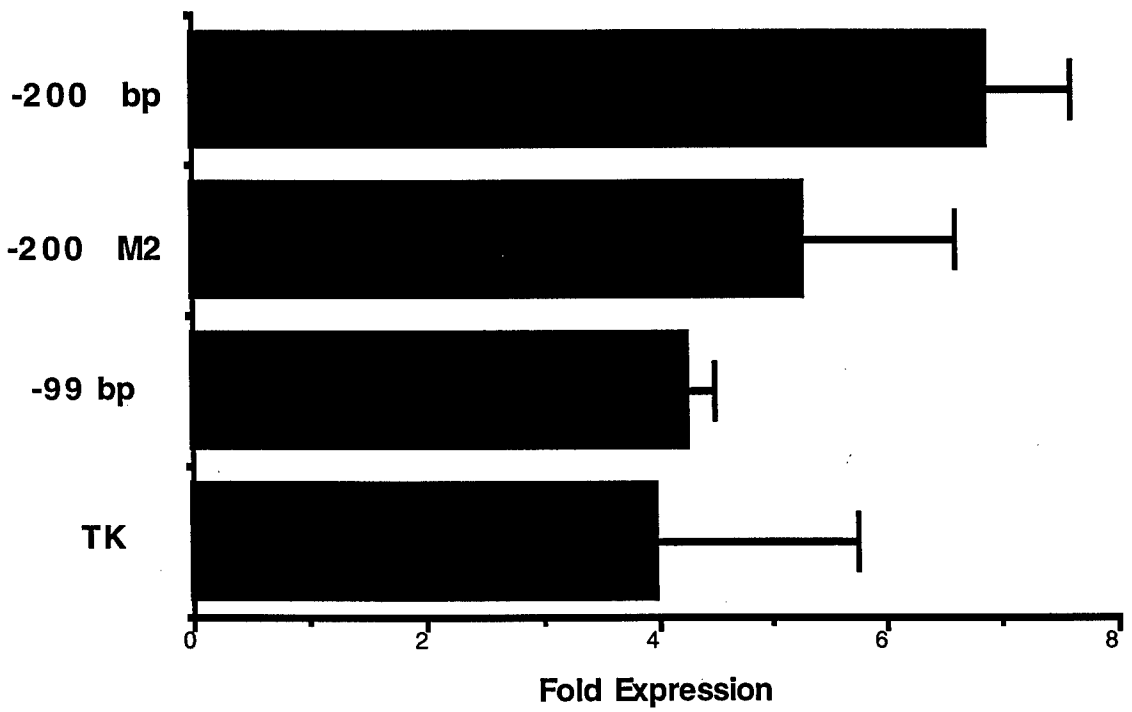
-200 bp
-200 M2
-99 bp
TK
0
2
4
6
8
Fold Expression

Figure 5

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Trans-Acting Factors Dictate the Species-Specific Placental Expression of Corticotropin-Releasing Factor Genes in Choriocarcinoma Cell Lines*

CAROLINE D. SCATENA AND STUART ADLER

Department of Obstetrics and Gynecology, Washington University School of Medicine (S.A.), and the Division of Biology and Biomedical Sciences, Program in Molecular and Cellular Biology (C.D.S., S.A.), Washington University, St. Louis, Missouri 63110

ABSTRACT

CRF, in addition to its role in the hypothalamus, demonstrates species-specific expression in the placentas of higher primates, but not rodents. Transient transfections of BeWo and JEG-3 choriocarcinoma cells, as models for human trophoblasts, demonstrate regulated expression of human (h) CRF-luciferase reporter genes, whereas little or no expression is detected in other lines, including CV-1 cells. The rodent choriocarcinoma cell line, Rcho-1, a model for rodent trophoblasts, is defective in the expression of transfected hCRF genes. The mouse CRF promoter behaves similarly to the corresponding hCRF construct. It is active in BeWo and inactive in Rcho-1 cells.

The transcriptional response to cAMP contributes to the specific expression of CRF. Analyses of deleted or mutated hCRF promoters identify a key role for protein kinase A-dependent pathways. A major part, but not all, of this effect is mediated by the canonical cAMP response element conserved in mouse, rat, and human CRF promoters. Additional deletions of the human CRF promoter identify control regions that also contribute to the observed species-specific expression pattern, and each identified region binds factors in nuclear extracts derived from the appropriate cell line. These studies using human and rodent choriocarcinoma cell lines as models of placental trophoblasts demonstrate dominant effects of cellular *trans*-acting factors, rather than DNA sequence differences, in dictating the species-specific placental expression of CRF. (*Endocrinology* **137:** 3000–3008, 1996)

THE HYPOTHALAMIC peptide CRF plays a key role in regulating the hypothalamic-pituitary-adrenal axis. CRF production is a critical first step in the synthesis of glucocorticoids, which are essential for life and an integral component of mammalian carbohydrate metabolism, and the stress response. The peptide sequence and expression of CRF are conserved across numerous animal species, indicating their importance in the maintenance of mammalian homeostasis (1). In addition to the hypothalamus, CRF is expressed in various peripheral tissues, including the placenta (1); however, its expression in this organ is uniquely species specific (2). The placentas of humans and higher primates express CRF messenger RNA (mRNA), whereas those of the rat, mouse, lemur, and guinea pig fail to express the gene (2, 3).

Recent studies indicate that CRF and its specific binding protein (4) act as a clock to time the onset of human labor (5). However, the precise role of placental CRF and its binding protein in the human physiology of fetal development and parturition has yet to be determined. From the seventh week of gestation until parturition, CRF mRNA is detected in human placenta (6). There is a gradual increase in expression of the gene during the course of a pregnancy; it increases dramatically during the last 5 weeks before delivery (6). Studies indicate that placental CRF may increase the production of PGs, known mediators of labor, and it may also potentiate the effect of oxytocin on uterine contractions (7). CRF produced in the placenta may enter the fetus and stimulate the fetal pituitary-adrenal axis, resulting in the increase in cortisol seen in fetal plasma during the last 5 weeks of pregnancy (8). The cortisol surge may allow proper maturation of fetal organs and serve as one of the signals necessary for the initiation of labor (8, 9). Alternatively, placental CRF may act in a paracrine fashion, stimulating the release of ACTH from the placenta (8), thereby influencing the fetal adrenal glands (8). CRF, acting as a lymphokine, might also modulate the immune relationship between the fetus and the mother (10).

Expression of CRF in placenta represents a distinct system for investigation of both cell type- and species-specific gene expression as well as a means to gain insight into the function of placental CRF in human physiology. In this work we have exploited the availability of human and rodent choriocarcinoma cell lines as models of placental trophoblasts together with promoter sequences from both the human and mouse CRF genes to investigate the molecular basis of the human-specific placental expression of CRF. Unlike previous studies of other human-specific placental genes, our results indicate a dominant role for species-specific *trans*-acting factors, rather than DNA sequence differences, in determining the expression pattern of the CRF gene.

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Address all correspondence and requests for reprints to: Dr. Stuart Adler, Department of Obstetrics and Gynecology, Washington University School of Medicine, 4911 Barnes Hospital Plaza, St. Louis, Missouri 63110-1094. E-mail: ADLER_S@WUMS.WUSTL.EDU.

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Materials and Methods

Luciferase plasmids

Luciferase reporter plasmids are constructed in a specially modified vector, LΔ2S. The vector is derived from pBLCAT2 (11), modified by replacing the chloramphenicol acetyltransferase (CAT) gene with a luciferase reporter, by incorporating simian virus 40 (SV40) termination signals upstream of the promoter, and by removing potential activator sites from both the plasmid backbone and the luciferase-coding region. The activating protein-1-related sequence aTGTGTC*A*ga, at nucleotides 1221–1232 of the luciferase gene (12), was replaced by aTGTGTG*C*ga using site-directed mutagenesis (13), a change that does not result in changes in the amino acid sequence. An identical aTGTGTCAga sequence in the pBLCAT2 backbone was removed by digestion with *Dra*II and *Nde*I. After replacing the CAT-SV40 region with the mutated luciferase-SV40 sequence, triplet termination signals (14) were inserted upstream of the promoter. This plasmid contains 5′-*Bam*HI and 3′-*Xho*I sites for insertion of promoter sequence cassettes.

Human and mouse CRF promoters

The human CRF genomic clone, SpHCRH-1, was the generous gift of Shosaku Numa (15). The 5-kilobase (kb) upstream region was isolated as a *Eco*RI-*Xho*I fragment after *Tth*III1 partial digestion (cutting at +13 bp), followed by filling and ligation to an *Xho*I linker. The 532-bp promoter was isolated by a complete *Tth*III1 digest. Additional deletions of the hCRF promoter were made by specific restriction digests or PCR. Deletions were confirmed by dideoxy sequencing.

The human (h) CRF-luciferase fusion construct contains the LΔ2S backbone and the 8-kb hCRF genomic clone. The mutated luciferase gene has been placed in-frame in the second exon, replacing the coding region of the hCRF prepropeptide. This construct contains 5 kb of 5′-promoter, the first exon and intron, the modified second exon encoding luciferase, and the 3′-flanking region.

The mouse CRF gene was isolated by screening a mouse genomic library using oligonucleotides contained in the 5′-proximal promoter and the second exon of the hCRF gene. After plaque purification of a single λ- mouse (m) CRF clone, the mCRF promoter was sequenced using the Promega PCR cycle sequencing kit (Promega Corp., Madison, WI). Oligonucleotides were designed to isolate the 536-bp promoter cassette by PCR. The 536-bp promoter was inserted into the LΔ2S luciferase vector.

Mutagenesis

Oligonucleotide-directed mutagenesis was performed in phagemid vectors using minor modifications of the method of Kunkel (13). The cAMP response element at −220 bp in the hCRF promoter was changed from TGACGTCA to GGAATTCC.

Expression vectors

Plasmids containing the Rous sarcoma virus (RSV) promoter for expressing the catalytic subunit of protein kinase A, Rous sarcoma virus-protein kinase A (RSV-PKA), and the heat-stable inhibitor of the cAMP-dependent protein kinase (PKI), RSV-PKI, were obtained from Richard Maurer (16, 17). The RSV-Neo plasmid, expressing the neomycin phosphotransferase II gene, was as previously described (18).

Cell lines

HeLa, CV-1, MDA-MB-231 (MB), BeWo, JAr, and JEG-3 cell lines were obtained from the American Type Culture Collection (Rockville, MD). In addition, a subclone of BeWo cells, b30, was the gift of A. Schwartz (19). BeWo cells from both sources behaved similarly in these experiments. Rcho-1, rat choriocarcinoma cells, were the generous gift of M. Soares (20). HeLa, CV-1, and MDA-MB-231 cells were grown in 10% CO_2 in DMEM with 5% FBS and 5% enriched calf serum (ECS; Gemini Bioproducts, Calabasa, CA). BeWo and Rcho-1 cells were grown in 5% CO_2 in NCTC-135 with 5% FBS, 5% ECS, 0.4% glucose, 50 μM 2-mercaptoethanol, and 100 μM sodium pyruvate. JEG-3 cells were grown in 5% CO_2 in MEM with 5% FBS and 5% ECS. JAR cells were grown in 5% CO_2 in RPMI 1640 with 10% FBS. All of the above growth media were supplemented with antibiotics. All cells are routinely surveyed for mycoplasma using a PCR method from Stratagene (La Jolla, CA).

Transfections

Transient transfections were performed using a calcium-phosphate method (21) in either 100-mm plates or 35-mm 6-well plates. Typically, for a 6-well plate, 100,000 cells/well were seeded in growth medium 2 days before transfection. On the day of transfection, cells were fed with DMEM containing 10% FBS and incubated in a 10% CO_2 environment. Four hours later, each 2-ml well was transfected with 150 μl *N,N*-bis (2-hydroxyethyl)-2-aminoethane-sulfonic acid (BES)-buffered saline (BBS)-$CaCl_2$ solution containing a total of 6 μg DNA. The DNA solution consisted of 3 μg luciferase reporter plasmid and salmon sperm DNA to bring the final DNA concentration to 6 μg. Alternatively, some experiments used 2.5 μg total DNA, with the same ratios of reporter and carrier DNA. Plates were then placed overnight in 5% CO_2. The next day, cells were rinsed with DMEM, fed their growth media, and hormone treated as indicated. One day after hormone treatment, cells were harvested in 150 μl of a Triton lysis buffer containing 50 mM Tris (hydroxymethyl)aminoethane, 50 mM 2(*N*-morpholino)ethane sulfonic acid (pH 7.8), 1 mM dithiothreitol, and 1% Triton X-100. The lysate was assayed for luciferase activity as previously described (22), using an Analytical Luminescence Laboratories (San Diego, CA) Monolight 2010 luminometer. β-Galactosidase assays were performed using chlorophenol red β-galactopyranoside (Boehringer Mannheim, Indianapolis, IN) as substrate (23) and an Anthos plate reader (Anthos Labtec Instruments, Salzburg, Austria) with Delta Soft II software (Bio Metallics, Princeton, NJ).

For transfection experiments, the data shown, when indicated, are luciferase values normalized to the basal activity of the herpes thymidine kinase promoter set at 100. Experiments determining the basal activity of CRF promoter constructs in BeWo and Rcho-1 cells showed indistinguishable results with or without inclusion of either pCH110 (Pharmacia, Piscataway, NJ) or an RSV β-galactosidase reporter and internal standardization. These β-galactosidase reporters are not neutral to hormone treatment, as we observed effects of forskolin, 8-bromo-cAMP (8-Br-cAMP), and PKA expression on β-galactosidase activity. In addition, we observed promoter interference that varied with the inherent strength or stimulated activity of each tested CRF luciferase deletion construct. For these reasons, all data presented were obtained from experiments without inclusion of additional β-galactosidase reporter plasmids for internal standardization.

Electromobility shift assays

Cell extracts from cultured cells were prepared using minor modifications of a microtechnique (24). DNA fragments were prepared by PCR and purified using PAGE. Probes were labeled using direct incorporation of radioactive nucleotides during PCR or with T4 polynucleotide kinase. For the human-specific activator, binding reactions contained 5–20 μg nuclear extract, binding buffer (25), 5 mM $MgCl_2$, 50 mM $NaPO_4$ (pH 7), and 5 μg poly(dI·dC)·poly(dI·dC). Cold competitor, when included, was at an approximately 100-fold molar excess. The final binding reaction volume, including probe, was 20 μl. Binding reactions were preincubated at room temperature for 10 min before the addition of probe. After probe addition, reactions were incubated overnight at 0 C to achieve binding equilibrium. Polyacrylamide gels (4% acrylamide-bis, 38:2) were electrophoresed at 4 C at 10 mA. Gels contained 2.5% glycerol and 0.5 × glycerol-tolerant gel buffer (Tris-Taurine-EDTA, TTE) (U.S. Biochemical Corp., Cleveland, OH). For the rodent activator, the binding reaction contained 5 or 10 μg nuclear extract. For the rodent repressor, the binding reactions contained 5 μg nuclear extract. Each reaction also contained 5 μg poly(dI-dC) and binding buffer containing 12% glycerol, 12 mM HEPES (pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, 20 μg/ml BSA, and 5 mM dithiothreitol. Cold competitor, when included, was at approximately a 100-fold molar excess. The final binding reaction volume, including probe, was 20 μl. Binding reactions were preincubated at room temperature for 10 min before the addition of probe. After probe addition, reactions were incubated for an additional 20 min before elec-

trophoresis. Polyacrylamide gels (4% acrylamide-bis, 80:1) were electrophoresed at 4 C at 1000 V. Gels contained 2.5% glycerol and 0.5 × TTE. Results were visualized using autoradiography at −80 C with an intensifying screen or by storage screen analysis.

Results

Human choriocarcinoma cell lines specifically express hCRF reporter genes

Choriocarcinoma cell lines have been widely used as a model of placental trophoblasts for studies of gene expression. These cell lines have been used in the analysis of placental gene expression, including CGα (19, 26–30) and been shown to contain *trans*-acting factors necessary for tissue-specific expression of this gene (26–29). We determined whether choriocarcinoma cells were an appropriate model for studying the expression of human CRF by analyzing these cells for expression and regulation of transfected hCRF luciferase reporter genes.

Two human choriocarcinoma cell lines, BeWo and JEG-3, were chosen as models for human placental trophoblasts. The cells were transfected with either of two luciferase reporter gene constructs (Fig. 1A). The first construct contains 5 kb of 5′-proximal hCRF sequence linked to the firefly luciferase reporter gene. The second construct uses the 8-kb hCRF genomic clone (15) in which the sequences encoding the CRF peptide were removed and replaced in-frame with the sequences encoding firefly luciferase (12). The construct retains the first exon, the intron, part of the second exon, the 3′-untranslated region, and the polyadenylation site from the human genomic clone. In transient transfection experiments, the expression of both constructs varied in different cell lines (Fig. 1B). In the human choriocarcinoma cell lines as well as the nonplacental CV-1 cells, basal levels of reporter gene expression were low (Fig. 1B). Upon stimulation of the PKA pathway with forskolin, the pattern of expression in the two cell types changed dramatically. Treatment of the human choriocarcinoma cell lines with forskolin resulted in an increase in CRF gene expression of approximately 8-fold, which did not occur in the nonplacental cell lines (Fig. 1B). Similar noninducible expression patterns were observed for the human HeLa and MB-231N nonplacental cells (data not shown), whereas a similar inducible pattern was observed for the human JAr choriocarcinoma cell line (data not shown).

The genomic fusion reporter gene displays lower basal levels of luciferase activity than the 5-kb promoter in CV-1 cells. This suggests that sequences outside the 5′-flanking region contribute to tissue-specific expression, in agreement with previous observations (31). However, the human choriocarcinoma-specific expression of the hCRF reporter gene

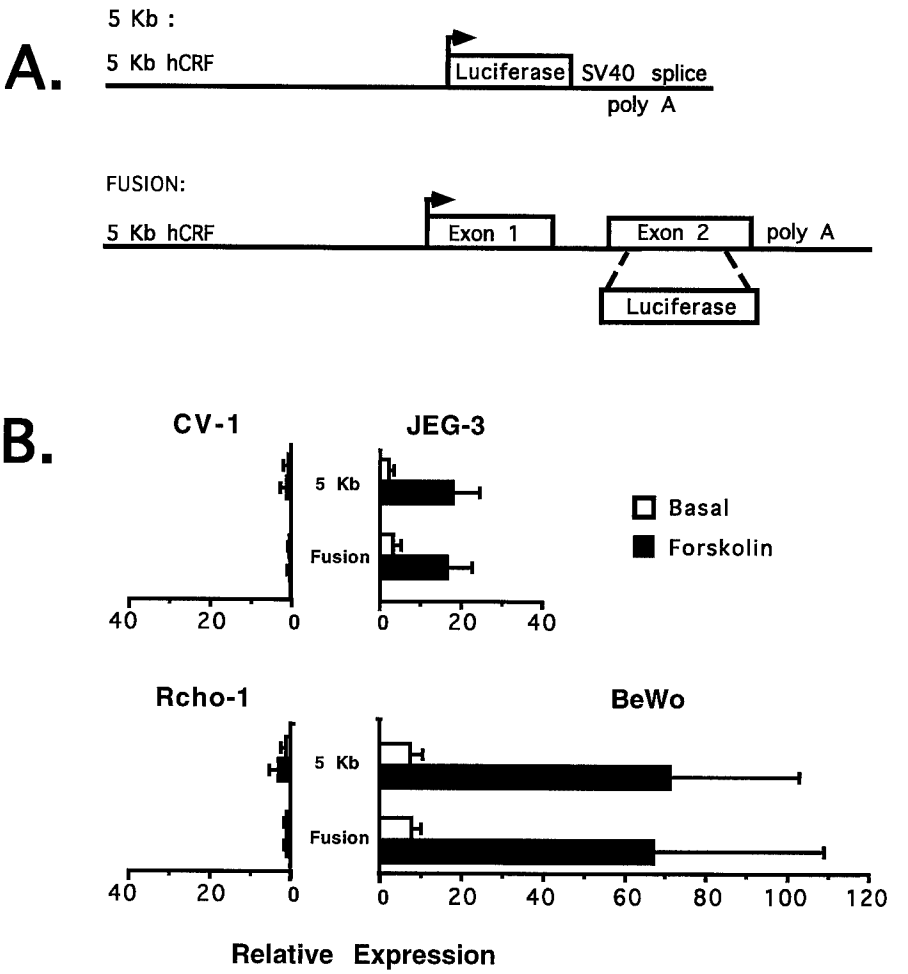

FIG. 1. A, hCRF luciferase reporter genes. The 5-kb CRF construct contains the 5′-flanking sequence of CRF fused to firefly luciferase, followed by the SV40 splice and polyadenlyation signal sequences. CRF-Fusion consists of the 8-kb hCRF genomic clone with the coding region for the CRF peptide removed and replaced with the sequence encoding firefly luciferase as an in-frame fusion. The *arrow* indicates the transcription start site. B, Cell type-specific expression of hCRF. JEG-3 and BeWo are human choriocarcinoma cell lines. CV-1 is a monkey kidney fibroblast line. Rcho-1 cells are a rat choriocarcinoma cell line. Using a standard calcium phosphate protocol (see *Materials and Methods*), the cells were transfected with the indicated reporter genes. Twenty-four hours posttransfection, cells received either vehicle or 25 μM forskolin; 24 h posthormone treatment, cells were harvested for luciferase assay (see *Materials and Methods*). Relative expression indicates luciferase values normalized to herpes thymidine kinase (TK) equal to 100. Results are the mean ± SEM from three experiments.

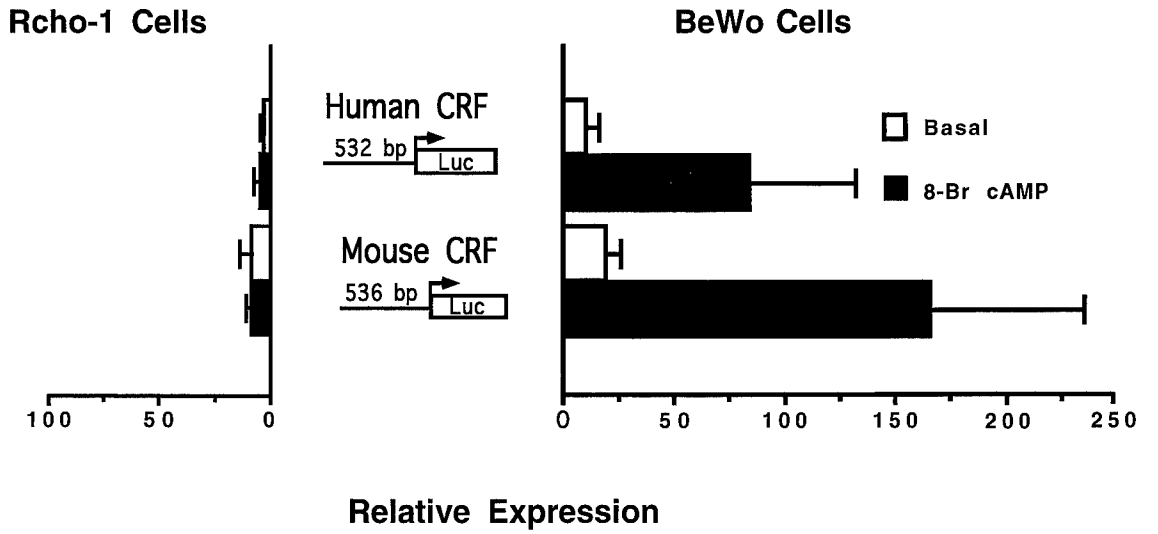

FIG. 2. Expression of human *vs.* mouse CRF in human and rodent choriocarcinoma cell lines. Human BeWo and rodent Rcho-1 cells were transfected with the indicated human or mouse CRF reporter genes. Twenty-four hours posttransfection, the indicated cells received 1 mM 8-Br-cAMP; 24 h posthormone treatment, cells were harvested for luciferase assay (see *Materials and Methods*). Relative expression indicates luciferase values normalized to herpes thymidine kinase (TK) equal to 100. Results are the mean ± SEM from three experiments.

containing only the 5′-flanking region indicates that *cis*-acting sequences within this region must also contribute to the observed tissue-specific expression pattern. Thus, the 5′-proximal promoter can be used in experiments to determine *cis*-acting sequences important for the species-specific expression of CRF in these cell lines. These results demonstrate that human choriocarcinoma cell lines are a suitable model for studying CRF expression and contain factors necessary for the regulated and specific expression of human CRF reporter genes.

Activity of the Rcho-1 cell line

The Rcho-1 cell line has been used as a model for previous studies of rodent trophoblast gene expression, including mouse placental lactogens I and II and P450SCC (20, 32, 33). We, therefore, performed a series of transient transfection experiments with CRF reporters using this cell line as a model for rodent placenta. There was little or no expression of the hCRF reporter genes in these cells, even after treatment for 24 h with forskolin (Fig. 1B). Because the same reporter constructs were effectively expressed in the human choriocarcinoma cell lines, the results suggest that *trans*-acting factors contribute to the lack of expression of CRF in this rodent choriocarcinoma cell model.

Expression of the mCRF gene in human and rodent cell lines

In addition to differing *trans*-acting factors that might control human and rodent CRF expression, DNA sequence differences might also contribute to this species-specific expression pattern. To determine whether DNA sequences contained in the mouse CRF gene might restrict its expression, a genomic clone of mouse CRF was isolated. Reporter gene constructs were made using 532 bp of the hCRF promoter and a corresponding 536-bp mouse CRF promoter. The two constructs were used in parallel in both BeWo and

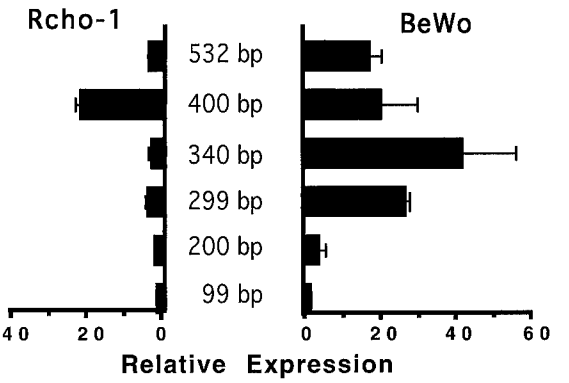

FIG. 3. Comparison of the basal expression of hCRF in human and rodent choriocarcinoma cell lines. Human BeWo and rodent Rcho-1 cells were transfected with the indicated reporters and harvested for luciferase assays (see *Materials and Methods*). Relative expression indicates luciferase values normalized to herpes thymidine kinase (TK) equal to 100. Results are the mean ± SEM of two experiments.

Rcho-1 cells. As shown in Fig. 2, in BeWo cells, both the human and mouse reporters were expressed and induced by the addition of 8-Br-cAMP. In contrast, in the Rcho-1 cell line, both constructs had lower basal expression and were not responsive to 8-Br-cAMP induction.

These results suggest that it is cellular factors differing between human and rodent choriocarcinoma cells rather than sequence differences between human and rodent CRF promoters that dictate the observed species-specific CRF expression. The results also imply that there are common *cis*-acting sequences that control CRF expression within the first 536 bp of mouse and human CRF 5′-flanking sequences.

Deletional analysis of the CRF promoter

To more precisely identify *cis*-acting sequences that participate in species- and cell type-specific CRF expression,

deletions of the human promoter were analyzed for expression. A series of chimeric luciferase reporter plasmids containing deletions of the human CRF 5′-proximal region was created using restriction enzyme digestion and PCR. Basal activities of this deletion series were determined in parallel in BeWo and Rcho-1 cells (Fig. 3). The different activities of each promoter in the human and rodent cell lines again show that *trans*-acting factors differing between human and rodent cells affect the species-specific expression of CRF (Fig. 3). Unlike the 532-bp promoter, which has low activity in Rcho-1 cells, the 400-bp CRF promoter displays higher basal expression in the rodent cells, essentially equivalent to the activity in human BeWo cells (Fig. 3). This suggests that factors interacting with the 132-bp segment from −532 to −400 limit the expression of the CRF promoter in the rodent cells. The 340-bp promoter shows similar elevated basal expression in BeWo cells, but decreased expression in the Rcho-1 cell line (Fig. 3). Factors interacting with the 60-bp sequence from −400 to −340 thus serve to activate expression of the hCRF gene in the rodent cell line. Further deletions of the hCRF promoter all displayed weak activity in Rcho-1 cells. In contrast, basal expression in BeWo cells remained elevated until removal of the region from −299 to −200. This region contains a canonical CRE. Although this 100-bp region may contain other important regulatory sites, these results suggest the potential importance of the CRE element in controlling basal as well as activated expression of CRF in human BeWo cells and indicate that the PKA pathway may be partially activated even under basal conditions.

Analysis of the PKA pathway

In an effort to determine whether the CRE is involved in regulating CRF gene expression, hCRF-luciferase constructs were created in which the canonical CRE element was mutated. The 5-kb X-CRE and 532-bp X-CRE luciferase constructs were transfected into BeWo cells (Fig. 4). The mutation of the CRE element resulted in a decrease in basal expression in BeWo cells (Fig. 4, *top*). Unexpectedly, although the CRE site was mutated, the X-CRE constructs still retained a 4-fold induction with 8-Br-cAMP (Fig. 4, *bottom*). This effect was seen not only in the 5-kb promoter, but also in the 532-bp

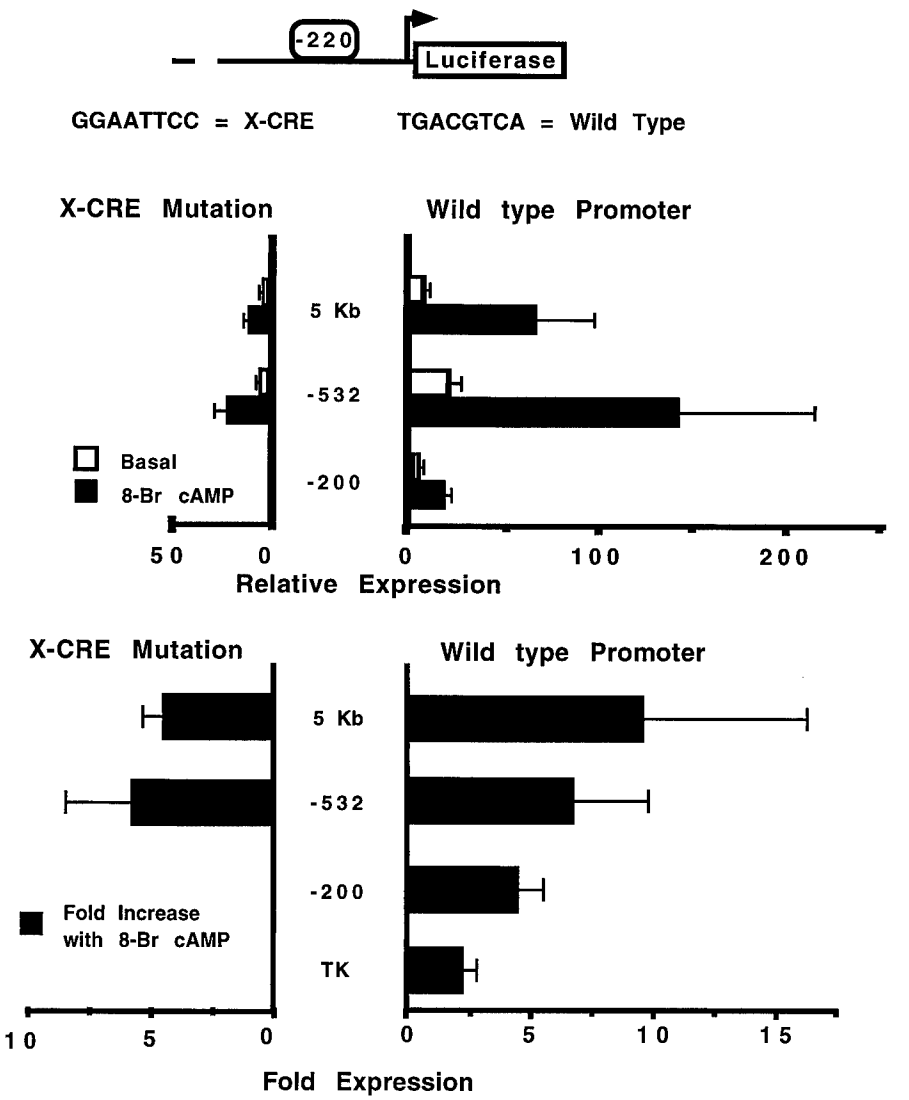

FIG. 4. Effect of the CRE element on CRF expression. X-CRE constructs containing the sequence GGAATTCC, which replaces the canonical CRE at −220, were created using site-directed mutagenesis. BeWo cells were transfected with wild-type or mutated promoters as indicated. Cells were treated with 1 mM 8-Br-cAMP 24 h after transfection and harvested after an additional 24 h. *Top*, Relative expression indicates luciferase values normalized to herpes thymidine kinase (TK) equal to 100. *Bottom*, Fold indicates the relative increase in expression with hormone treatment for each promoter construct. Data are the mean ± SEM from three experiments.

promoter and even in the 200-bp CRF promoter, which lacks the canonical CRE sequence (located at −220). In contrast, only a 2-fold induction was seen with the control thymidine kinase promoter (Fig. 4, *bottom*). Comparison of the fold activation of the 5-kb, −532, and −200 promoters (Fig. 4, *bottom*) as well as additional deletion constructs of the hCRF promoter with or without a mutated CRE (data not shown) suggests that several promoter regions may contribute to this CRE-like effect. This effect occurs in human BeWo cells, but is not observed in Rcho-1 cells (data not shown) and, thus, is species specific. Similar preservation of cAMP responsiveness was observed with forskolin treatment or direct activation by cotransfecting the PKA catalytic β-subunit (17) (data not shown). These results indicate that either there is a variant CRE element not yet identified within the −200 5′-flanking sequence, or there is another *trans*-acting factor influenced by the PKA pathway that interacts with these regions. The cAMP regulatory pathway is known to be very important in controlling the expression of placental genes (34). It appears that this regulatory pathway is involved in the control of the CRF gene in human BeWo cells.

Species-specific trophoblast factors bind to the hCRF promoter

The experiments presented above suggest that trophoblast *trans*-acting factors, rather than DNA sequence differences, dictate the species-specific expression of CRF. Additionally, deletional analysis of the human promoter has identified potential regulatory sites. To study this further, we performed gel shift assays with nuclear extracts from human JEG-3 and BeWo cells and rodent Rcho-1 cells, using the DNA promoter sequences that contribute to species-specific expression identified by transfectional analyses. Figure 5 shows the results of a comparison of nuclear extracts binding to a 82-bp fragment of the hCRF gene from −150 to −68 that contains sequences responsive to PKA stimulation in BeWo cells. A common, low band is seen in extracts from both human cell lines, JEG-3 and BeWo (Fig. 5, *left panel*), specifically competed by excess unlabeled fragment (Fig. 5, *right panel*). Notably, this band is reduced or absent in the Rcho-1 extract, although extracts bind this fragment with a different mobility (Fig. 5, *left panel*). These results further support the conclusion that *trans*-acting factors differing between rodent and human choriocarcinoma/trophoblast cells are responsible for dictating the species-specific pattern of placental CRF gene expression.

In addition to the human-specific PKA-responsive activator, two other regions of interest were identified by transfectional mapping. A repressor sequence was identified in Rcho-1 cells at −532 to −400 bp that when removed increases expression of the hCRF promoter. Gel shift analysis showed that factors present in Rcho-1 cells bound this region and were specifically competed by excess unlabeled fragment (Fig. 6, *left panel*). Although extracts from BeWo cells also bound this fragment, they exhibited a different mobility (data not shown). Transfections in the Rcho-1 cell line also identified a sequence at −400 to −340 bp that increased expression. Gel shift analysis showed a factor present in Rcho-1 cells that bound this region and was specifically competed with excess unlabeled fragment (Fig. 6, *right panel*). Whether this activator may participate in the regulation of other rodent-specific placental genes has yet to be determined.

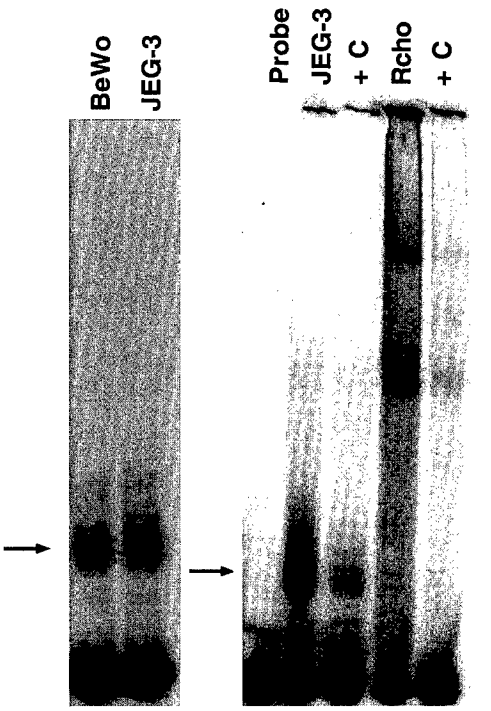

FIG. 5. Electromobility shift analyses of the hCRF promoter. The conditions for the assays are described in *Materials and Methods*. The *arrows* indicate the locations of the shifted bands of interest. The probe is from −150 to −68 bp of the hCRF gene. *Left panel*, Extracts from human choriocarcinoma cell lines, BeWo and JEG-3, display similar shifts. BeWo, Nuclear extract from BeWo cells; JEG-3, nuclear extract from JEG-3 cells. *Right panel*, Human and rodent choriocarcinoma cells display different shifts. Probe, Probe alone; JEG-3, extract from JEG-3 cells; +C, JEG-3 cell extract plus excess unlabeled competitor; Rcho, extract from Rcho-1 cells; +C, Rcho-1 cell extract plus excess unlabeled competitor.

Discussion

CRF exhibits a complex pattern of expression and regulation. In the hypothalamus, CRF regulates the expression and release of ACTH from the anterior pituitary (1). CRF in the hypothalamus is expressed in a circadian pattern (35). It is subject to feedback inhibition at the level of transcription by glucocorticoids, the end product of the hypothalamic-pituitary-adrenal axis that CRF controls (36). CRF is also a key part of the stress response, with high levels of CRF stimulating increased levels of glucocorticoids, but in a manner that is not sensitive to feedback inhibition (1). CRF is expressed by T lymphocytes (3) and may itself act as a lymphokine, directly influencing the immune system independent of ACTH (10). Placental expression of CRF is primate specific (2), and CRF plays a role in timing the onset of human labor (5). Yet, the regulators of CRF expression in human trophoblasts and the mechanisms used by CRF to affect maternal-fetal physiology and parturition are not well understood. In placenta, expression of CRF increases throughout gestation (6) and is neither circadian nor feedback regulated by glucocorticoids (8). CRF is a single copy gene (37), and the

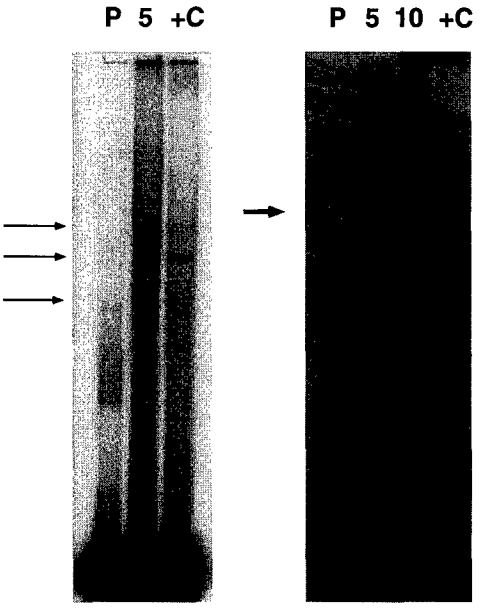

FIG. 6. Electromobility shift analyses of the hCRF promoter. The conditions for the assays are described in *Materials and Methods*. The *arrows* indicate the locations of the shifted bands of interest. *Left panel*, CRF repressor. The probe is from −532 to −400 bp of the hCRF gene. P, Probe alone; 5, 5 μg extract from Rcho-1 cells; +C, Rcho-1 cell extract plus excess unlabeled competitor. *Right panel*, Rodent CRF activator. The probe is from −400 to −340 bp of the hCRF gene. P, Probe alone; 5, 5 μg extract from Rcho-1 cells; 10, 10 μg extract from Rcho-1 cells; +C, 10 μg Rcho-1 cell extract plus excess unlabeled competitor.

peptide and proximal promoter sequences are highly conserved in the human, rat, and mouse genes (15, 38–40). Furthermore, CRF mRNA is identical in the human hypothalamus and placenta (41), suggesting that despite the differences in regulated expression, at least some promoter sequences may be used in both tissues.

There are currently no suitable cell culture systems available for detailed molecular studies of the regulation of CRF in hypothalamic neurons. However, there are several useful model cell lines for human placental trophoblasts. The JAR, JEG-3, and BeWo choriocarcinoma cell lines retain features of placental trophoblasts and have been successfully used for molecular studies of placental gene expression (19, 26–30). Recently, a corresponding rodent choriocarcinoma cell line, Rcho-1, has been established and used for expression studies (20). The combination of choriocarcinoma cell lines and CRF promoter fragments from both the human and mouse have provided the tools to directly compare the expressions of both genes in cells of human or rodent origin. Our experiments investigate the basis of species-specific expression of CRF by segregating the contributions of species-specific *trans*-acting factors from *cis*-acting sequences (Fig. 7). These results consistently demonstrate that *trans*-acting factors, differing between human and rodent choriocarcinoma cell lines, play a dominant role in dictating the species-specific expression pattern of CRF. Our results in these model systems reflect the results observed in placental trophoblasts. In human placenta, the hCRF gene is expressed, and in the human BeWo cell line model, hCRF reporter genes are expressed. In contrast, in rodent placenta, the CRF gene is not expressed, and in the Rcho-1 rodent cell line model, a mouse CRF reporter gene is not expressed. The experiments in which rodent and human reporter genes are expressed in cells derived from the heterologous species allow determination of the contributions of cellular *vs.* sequence differences. The human BeWo cells express a mouse CRF reporter gene, whereas rodent Rcho-1 cells do not express hCRF reporter genes. Therefore, *trans*-acting factors differing between human and rodent cells dominate in the observed species-specific CRF expression pattern in choriocarcinoma cells.

The results from these studies differ significantly from those reported for CG, which has served as a model for human-specific placental expression. The α-subunit of CG, like CRF, demonstrates a species-specific expression pattern. It is expressed in the placentas of humans, higher primates, and horses, whereas expression is restricted to the pituitary in other animal species (26). In humans and primates, tissue-specific expression of CGα results from a combination of *cis*-acting elements located in the 5′-proximal promoter region (26–29). In humans, two 18-bp direct repeats exist that contain a consensus cAMP response element (CRE), TGACGTCA (27, 28, 42, 43). The two repeats are absolutely necessary for cAMP responsiveness as well as tissue-specific expression (27, 28, 42, 43). Upon deleting these repeats, placenta-specific expression of a CAT reporter gene driven by the CGα promoter decreases to background levels (27). Upstream of the repeats, another *cis*-acting element exists that confers placenta-specific expression to a nonplacental heterologous promoter (27). It is known as the trophoblast specific element (TSE), and it binds a placenta-specific protein, TSEB (27, 29). If one deletes the CREs, the TSE loses its activity (27). Therefore, it appears that a protein-protein interaction occurs between a CRE-binding protein family member bound to the CREs and the TSEB, and the interaction is required for placenta-specific expression (28). In primates, placental expression of CGα depends on the presence of the TSE and either one or two copies of the 18-bp CRE (26). Thus, it appears that the species-specific expression of CGα in the placenta is very complex, relying on a combination of various *cis*-acting elements located upstream of the TATA box.

The species-specific expression of CRF, unlike that of CGα, cannot depend on the differences in CRE sequences, as functional CREs are conserved in human, mouse, and rat promoters (15, 30, 38, 44). Furthermore, our experiments comparing mouse and human CRF promoters in the choriocarcinoma cell lines confirm the dominant effect of species-specific *trans*-acting factors in determining the placental expression of CRF. Nonetheless, the CRE and the factors mediating this response appear to play a major role in CRF expression.

cAMP pathways play an important role in the biology of placental trophoblasts (34). In addition to the canonical sequences in CG and CRF, cAMP may play a critical role in the developmental pathway of trophoblasts, modulating both the morphological and biochemical changes that occur during the differentiation of cytotrophoblasts to syncytial trophoblasts (19). The induction of transcription factors by cAMP may play an important role in mediating its effects. Other endocrine genes including LHβ are regulated by cAMP, but seem to lack canonical CRE sequences (45). Our data demonstrate CRF induction via the CRE, but do not exclude the participation of additional sequences. We have

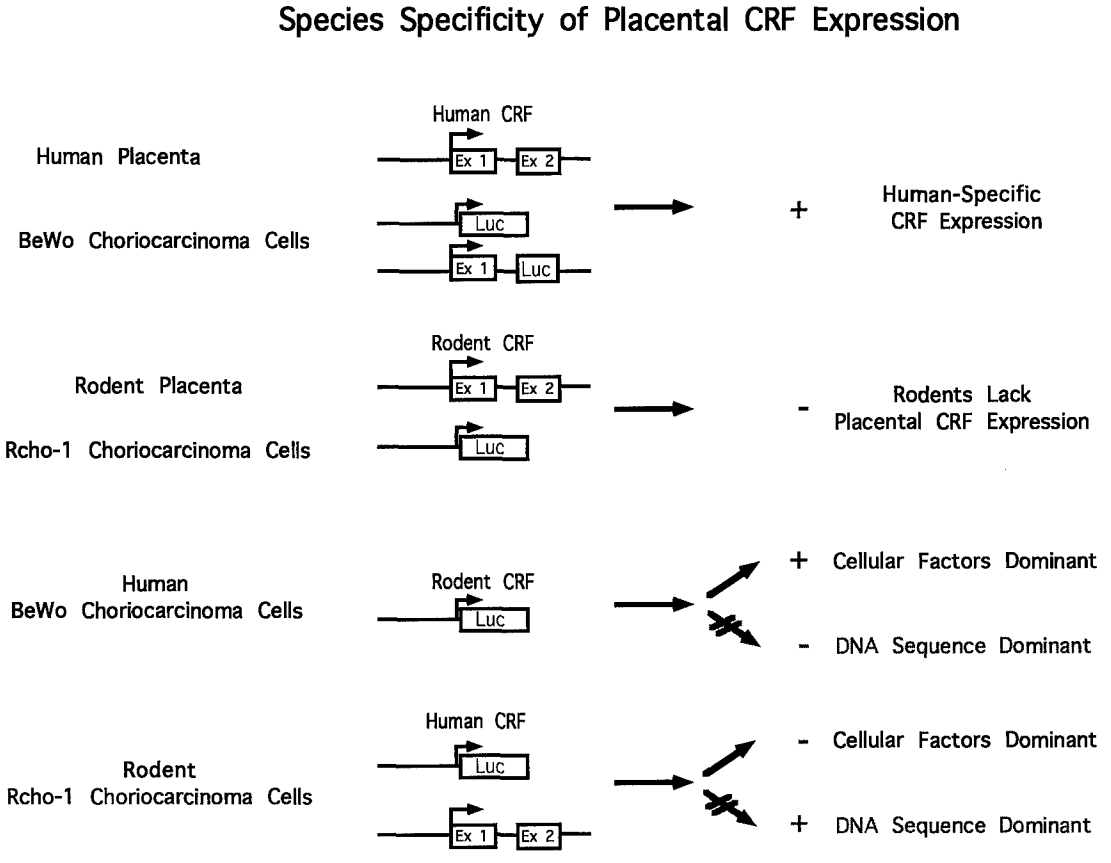

FIG. 7. Species specificity of placental CRF expression. The species specificity of CRF expression in placenta was modelled using human and rat choriocarcinoma cell lines and reporter genes derived from both human and mouse CRF genes. In human placenta and BeWo cells, hCRF genes are expressed (+). In rodent placenta and Rcho-1 cells, rodent CRF genes are not expressed (−). The basis of this species difference in CRF expression can be explained by either differences in cellular factors (Cellular Factors Dominant) or DNA sequence differences (DNA Sequence Dominant). Introduction of human and rodent CRF reporter genes into cells from the other species distinguishes these two possibilities. The results presented above are consistent with the former alternative in determining species-specific CRF expression.

identified one such region proximal to −200 bp, and a corresponding candidate nuclear binding factor in both BeWo and JEG-3 cells. We have no information yet whether this non-CRE-mediated activation may reflect synergy, modification, or activation of preexisting factors, or whether cAMP initiates a transcription cascade ultimately leading to the production of new transcriptionally active protein factors.

Two additional sites were located in the CRF 5′-flanking promoter that are involved in regulating CRF expression. A repressive sequence from −532 to −400 bp prevents the expression of CRF in Rcho-1 cells. The effect of this sequence is on basal expression. This indicates that the repression of CRF in rodent trophoblasts cannot be solely due to differences in the ability of Rcho-1 cells to respond to forskolin and 8-Br-cAMP, but also involves distinct *trans*-acting factors separate from these responses. We have identified a candidate nuclear factor that binds to this region that is present in Rcho-1 cells. In addition to the repressive element, a positive *cis*-acting element was identified within the region from −400 to −340 bp. The ability of this element to increase the basal expression of CRF in the rodent trophoblast is unmasked by removal of the upstream repressor sequences. Although the factor interacting with this sequence does not mediate expression of the full CRF promoter, a role for this factor in the regulation of other rodent trophoblast genes has not yet been investigated.

The participation of all of these CRF sequences and factors in central nervous system expression in paraventricular hypothalamic parvocellular neurons or the activity of potential repressors in non-CRF-expressing neurons remains to be determined. Also, the identities of the trophoblast factors that bind to these sequences and their potential roles in the regulation of other placental genes and in human development and parturition remain to be elucidated by future studies.

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